

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, C07H 21/04, C12P 19/34		A1	(11) International Publication Number: WO 97/46705 (43) International Publication Date: 11 December 1997 (11.12.97)
(21) International Application Number: PCT/US97/09533 (22) International Filing Date: 3 June 1997 (03.06.97)		(81) Designated States: CA, IL, JP, Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 08/656,716 3 June 1996 (03.06.96) US 08/835,728 11 April 1997 (11.04.97) US		Published <i>With international search report.</i>	
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(54) Title: METHYLATION SPECIFIC DETECTION

(57) Abstract

The present invention provides a method of PCR, methylation specific PCR (MSP), for rapid identification of DNA methylation patterns in a CpG-containing nucleic acid. MSP uses the PCR reaction itself to distinguish between methylated and unmethylated DNA, which adds an improved sensitivity of methylation detection.

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METHYLATION SPECIFIC DETECTION

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Field of the Invention

The present invention relates generally to regulation of gene expression, and more specifically to a method of determining the DNA methylation status of CpG 10 sites in a given locus.

Background of the Invention

In higher order eukaryotes DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide. This modification has important regulatory 15 effects on gene expression, especially when involving CpG rich areas, known as CpG islands, located in the promoter regions of many genes. While almost all gene-associated islands are protected from methylation on autosomal chromosomes, extensive methylation of CpG islands has 20 been associated with transcriptional inactivation of selected imprinted genes and genes on the inactive X - chromosome of females. Aberrant methylation of normally unmethylated CpG islands has been described as a frequent 25 event in immortalized and transformed cells, and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers.

Human cancer cells typically contain somatically altered genomes, characterized by mutation, amplification, or deletion of critical genes. In 30 addition, the DNA template from human cancer cells often displays somatic changes in DNA methylation (E.R. Fearon, et al., *Cell*, 61:759, 1990; P.A. Jones, et al., *Cancer*

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Res., 46:461, 1986; R. Holliday, *Science*, 238:163, 1987; A. De Bustros, et al., *Proc. Natl. Acad. Sci., USA*, 85:5693, 1988); P.A. Jones, et al., *Adv. Cancer Res.*, 54:1, 1990; S.B. Baylin, et al., *Cancer Cells*, 3:383, 5 1991; M. Makos, et al., *Proc. Natl. Acad. Sci., USA*, 89:1929, 1992; N. Ohtani-Fujita, et al., *Oncogene*, 8:1063, 1993). However, the precise role of abnormal DNA methylation in human tumorigenesis has not been established. DNA methylases transfer methyl groups from the 10 universal methyl donor S-adenosyl methionine to specific sites on the DNA. Several biological functions have been attributed to the methylated bases in DNA. The most established biological function is the protection of the DNA from digestion by cognate restriction enzymes. The 15 restriction modification phenomenon has, so far, been observed only in bacteria. Mammalian cells, however, possess a different methylase that exclusively methylates cytosine residues on the DNA, that are 5' neighbors of guanine (CpG). This methylation has been shown by 20 several lines of evidence to play a role in gene activity, cell differentiation, tumorigenesis, X-chromosome inactivation, genomic imprinting and other major biological processes (Razin, A., H., and Riggs, R.D. eds. in *DNA Methylation Biochemistry and Biological 25 Significance*, Springer-Verlag, New York, 1984).

A CpG rich region, or "CpG island", has recently been identified at 17p13.3, which is aberrantly hypermethylated in multiple common types of human cancers (Makos, M., et al., *Proc. Natl. Acad. Sci. USA*, 89:1929, 30 1992; Makos, M., et al., *Cancer Res.*, 53:2715, 1993; Makos, M., et al., *Cancer Res.* 53:2719, 1993). This hypermethylation coincides with timing and frequency of 17p losses and p53 mutations in brain, colon, and renal

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cancers. Silenced gene transcription associated with hypermethylation of the normally unmethylated promoter region CpG islands has been implicated as an alternative mechanism to mutations of coding regions for inactivation 5 of tumor suppressor genes (Baylin, S.B., et al., *Cancer Cells*, 3:383, 1991; Jones, P.A. and Buckley, J.D., *Adv. Cancer Res.*, 54:1-23, 1990). This change has now been associated with the loss of expression of VHL, a renal cancer tumor suppressor gene on 3p (J.G. Herman, et al., 10 *Proc. Natl. Acad. Sci. USA*, 91:9700-9704, 1994), the estrogen receptor gene on 6q (Ottaviano, Y.L., et al., *Cancer Res.*, 54:2552, 1994) and the H19 gene on 11p (Steenman, M.J.C., et al., *Nature Genetics*, 7:433, 1994).

In eukaryotic cells, methylation of cytosine 15 residues that are immediately 5' to a guanosine, occurs predominantly in CG poor regions (Bird, A., *Nature*, 321:209, 1986). In contrast, discrete regions of CG dinucleotides called CpG islands remain unmethylated in normal cells, except during X-chromosome inactivation 20 (Migeon, et al., *supra*) and parental specific imprinting (Li, et al., *Nature*, 366:362, 1993) where methylation of 5' regulatory regions can lead to transcriptional repression. *De novo* methylation of the Rb gene has been demonstrated in a small fraction of retinoblastomas 25 (Sakai, et al., *Am. J. Hum. Genet.*, 48:880, 1991), and recently, a more detailed analysis of the VHL gene showed aberrant methylation in a subset of sporadic renal cell carcinomas (Herman, et al., *Proc. Natl. Acad. Sci.*, U.S.A., 91:9700, 1994). Expression of a tumor suppressor 30 gene can also be abolished by *de novo* DNA methylation of a normally unmethylated 5' CpG island (Issa, et al., *Nature Genet.*, 7:536, 1994; Herman, et al., *supra*; Merlo, et al., *Nature Med.*, 1:686, 1995; Herman, et al., *Cancer*

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Res., 56:722, 1996; Graff, et al., Cancer Res., 55:5195, 1995; Herman, et al., Cancer Res., 55:4525, 1995).

Most of the methods developed to date for detection of methylated cytosine depend upon cleavage of 5 the phosphodiester bond alongside cytosine residues, using either methylation-sensitive restriction enzymes or reactive chemicals such as hydrazine which differentiate between cytosine and its 5-methyl derivative. The use of methylation-sensitive enzymes suffers from the 10 disadvantage that it is not of general applicability, since only a limited proportion of potentially methylated sites in the genome can be analyzed. Genomic sequencing protocols which identify a 5-MeC residue in genomic DNA as a site that is not cleaved by any of the Maxam-Gilbert 15 sequencing reactions, are a substantial improvement on the original genomic sequencing method, but still suffer disadvantages such as the requirement for large amount of genomic DNA and the difficulty in detecting a gap in a sequencing ladder which may contain bands of varying 20 intensity.

Mapping of methylated regions in DNA has relied primarily on Southern hybridization approaches, based on the inability of methylation-sensitive restriction enzymes to cleave sequences which contain one 25 or more methylated CpG sites. This method provides an assessment of the overall methylation status of CpG islands, including some quantitative analysis, but is relatively insensitive, requires large amounts of high molecular weight DNA and can only provide information 30 about those CpG sites found within sequences recognized by methylation-sensitive restriction enzymes. A more sensitive method of detecting methylation patterns combines the use of methylation-sensitive enzymes and the

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polymerase chain reaction (PCR). After digestion of DNA with the enzyme, PCR will amplify from primers flanking the restriction site only if DNA cleavage was prevented by methylation. Like Southern-based approaches, this 5 method can only monitor CpG methylation in methylation-sensitive restriction sites. Moreover, the restriction of unmethylated DNA must be complete, since any uncleaved DNA will be amplified by PCR yielding a false positive result for methylation. This approach has been useful in 10 studying samples where a high percentage of alleles of interest are methylated, such as the study of imprinted genes and X-chromosome inactivated genes. However, difficulties in distinguishing between incomplete restriction and low numbers of methylated alleles make 15 this approach unreliable for detection of tumor suppressor gene hypermethylation in small samples where methylated alleles represent a small fraction of the population.

Another method that avoids the use of restriction 20 endonucleases utilizes bisulfite treatment of DNA to convert all unmethylated cytosines to uracil. The altered DNA is amplified and sequenced to show the methylation status of all CpG sites. However, this method is technically difficult, labor intensive and without 25 cloning amplified products, it is less sensitive than Southern analysis, requiring approximately 10% of the alleles to be methylated for detection.

Identification of the earliest genetic changes in tumorigenesis is a major focus in molecular cancer 30 research. Diagnostic approaches based on identification of these changes are likely to allow implementation of early detection strategies and novel therapeutic

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approaches targeting these early changes might lead to more effective cancer treatment.

Summary of the Invention

The precise mapping of DNA methylation patterns in 5 CpG islands has become essential for understanding diverse biological processes such as the regulation of imprinted genes, X-chromosome inactivation, and tumor suppressor gene silencing in human cancer. The present invention provides a method for rapid assessment of the 10 methylation status of any group of CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes. Despite the knowledge of those of skill in the art regarding the use of PCR and the use of bisulfite modification, independently, until the present 15 invention, no one had prepared primers that were specific for the bisulfite reaction such that the PCR reaction itself was used to distinguish between the chemically modified methylated and unmethylated DNA.

The method of the invention includes modification 20 of DNA by sodium bisulfite or a comparable agent which converts all unmethylated but not methylated cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA. This method of "methylation specific PCR" or MSP, requires 25 only small amounts of DNA, is sensitive to 0.1 % of methylated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples, for example. MSP eliminates the false positive results inherent to previous PCR-based approaches which 30 relied on differential restriction enzyme cleavage to distinguish methylated from unmethylated DNA.

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In a particular aspect of the invention, MSP is useful for identifying promoter region hypermethylation changes associated with transcriptional inactivation in tumor suppressor genes, for example, p16, p15, E-cadherin 5 and VHL, in human neoplasia. Other genes that are shown to be methylated include the estrogen receptor, MDGI, GST-pi, calcitonin, HIC-1, endothelin B receptor, TIMP-2, 06-MGMT, MLH1, MSH2, and GFAP. Of those, the estrogen receptor, MDGI, GST-pi, calcitonin, HIC-1, endothelin B 10 receptor, TIMP-2, 06-MGMT, and MLH1 were shown by MSP to be hypermethylated in neoplastic tissue as compared with normal tissue. For the first time, the invention provides evidence that TIMP-2, a tissue inhibitor of metalloproteinases, is hypermethylated in neoplastic 15 tissue as compared with normal tissue.

Brief Description of the Drawings

Figure 1 shows genomic sequencing of p16. The sequence shown has the most 5' region at the bottom of the gel, beginning at +175 in relation to a major 20 transcriptional start site (Hara, et al., *Mol. Cell Biol.*, 16:859, 1996). All cytosines in the unmethylated cell line H249 have been converted to thymidine, while all C's in CpG dinucleotides in the methylated cell H157 remains as C, indicating methylation.] enclosed a *Bst*UI 25 site which is at -59 in relation to the transnational start site in Genbank sequence U12818 (Hussussian, et al., *Nat. Genet.*, 8:15, 1994), but which is incorrectly identified as CGCA in sequence X94154 (Hara, et al., *supra*). This CGCG site represents the 3' location of the 30 sense primer used for p16 MSP.

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Figure 2, panels A-E, show polyacrylamide gels with the Methylation Specific PCR products of p16. Primer sets used for amplification are designated as unmethylated (U), methylated (M), or unmodified/wild-type (W). * designates the molecular weight marker pBR322-*MspI* digest. Panel A shows amplification of bisulfite-treated DNA from cancer cell lines and normal lymphocytes, and untreated DNA (from cell line H249). Panel B shows mixing of various amount of H157 DNA with 1 μ g of H249 DNA prior to bisulfite treatment to assess the detection sensitivity of MSP for methylated alleles. Modified DNA from a primary lung cancer sample and normal lung are also shown. Panel C shows amplification with the p16-U2 (U) primers, and p16-M2 (M) described in Table 1. Panel D shows the amplified p16 products of panel C restricted with *BstUI*(+) or not restricted (-). Panel E shows results of testing for regional methylation of CpG islands with MSP, using sense primers p16-U2 (U) and p16-M2 (M), which are methylation specific, and an antisense primer which is not methylation specific.

Figure 3, panels A-E, show polyacrylamide gels of MSP products from analysis of several genes. Primer sets used for amplification are not designated as unmethylated (U), methylated (M), or unmodified/wild-type (W). * designates the molecular weight marker pBR322-*MspI* digest and ** designates the 123bp molecular weight marker. All DNA samples were bisulfite treated except those designated untreated. Panel A shows the results from MSP for p15. Panel B shows the p15 products restricted with *BstUI*(+) or not restricted (-). Panel C shows the products of MSP for VHL. Panel D shows the VHL products restricted with *BstUI*(+) or not restricted (-). Panel E shows the products of MSP for *E-cadherin*.

Description of the Preferred Embodiments

The present invention provides methylation specific PCR (MSP) for identification of DNA methylation patterns. MSP uses the PCR reaction itself to distinguish 5 between modified methylated and unmethylated DNA, which adds an improved sensitivity of methylation detection.

Unlike previous genomic sequencing methods for methylation identification which utilizes amplification primers which are specifically designed to avoid the CpG 10 sequences, MSP primers themselves are specifically designed to recognize CpG sites to take advantage of the differences in methylation to amplify specific products to be identified by the invention assay.

As illustrated in the Examples below, MSP provides 15 significant advantages over previous PCR and other methods used for assaying methylation. MSP is markedly more sensitive than Southern analyses, facilitating detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows the study of 20 paraffin-embedded materials, which could not previously be analyzed by Southern analysis. MSP also allows examination of all CpG sites, not just those within sequences recognized by methylation-sensitive restriction enzymes. This markedly increases the number of such sites 25 which can be assessed and will allow rapid, fine mapping of methylation patterns throughout CpG rich regions. MSP also eliminates the frequent false positive results due to partial digestion of methylation-sensitive enzymes inherent in previous PCR methods for detecting 30 methylation. Furthermore, with MSP, simultaneous detection of unmethylated and methylated products in a single sample confirms the integrity of DNA as a template

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for PCR and allows a semi-quantitative assessment of allele types which correlates with results of Southern analysis. Finally, the ability to validate the amplified product by differential restriction patterns is an 5 additional advantage.

The only technique that can provide more direct analysis than MSP for most CpG sites within a defined region is genomic sequencing. However, MSP can provide similar information and has the following advantages.

10 First, MSP is much simpler and requires less than genomic sequencing, with a typical PCR and gel analysis taking 4-6 hours. In contrast, genomic sequencing, amplification, cloning, and subsequent sequencing may take days. MSP also avoids the use of expensive sequencing reagents and 15 the use of radioactivity. Both of these factors make MSP better suited for the analysis of large numbers of samples. Third, the use of PCR as the step to distinguish methylated from unmethylated DNA in MSP allows for significant increase in the sensitivity of methylation 20 detection. For example, if cloning is not used prior to genomic sequencing of the DNA, less than 10% methylated DNA in a background of unmethylated DNA cannot be seen (Myohanen, et al., *supra*). The use of PCR and cloning does allow sensitive detection of methylation patterns in 25 very small amounts of DNA by genomic sequencing (Frommer, et al., *Proc. Natl. Acad. Sci. USA*, 89:1827, 1992; Clark, et al., *Nucleic Acids Research*, 22:2990, 1994). However, this means in practice that it would require sequencing analysis of 10 clones to detect 10% methylation, 100 30 clones to detect 1% methylation, and to reach the level of sensitivity we have demonstrated with MSP (1:1000), one would have to sequence 1000 individual clones.

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In a first embodiment, the invention provides a method for detecting a methylated CpG-containing nucleic acid, the method including contacting a nucleic acid-containing specimen with an agent that modifies 5 unmethylated cytosine, amplifying the CpG-containing nucleic acid in the specimen by means of CpG-specific oligonucleotide primers, wherein the oligonucleotide primers distinguish between modified methylated and non-methylated nucleic acid and detecting the methylated 10 nucleic acid. It is understood that while the amplification step is optional, it is desirable in the preferred method of the invention. The method of the invention relies on the PCR reaction itself to distinguish between modified (e.g., chemically modified) 15 methylated and unmethylated DNA.

The term "modifies" as used herein means the conversion of an unmethylated cytosine to another nucleotide which will distinguish the unmethylated from the methylated cytosine. Preferably, the agent modifies 20 unmethylated cytosine to uracil. Preferably, the agent used for modifying unmethylated cytosine is sodium bisulfite, however, other agents that similarly modify unmethylated cytosine, but not methylated cytosine can also be used in the method of the invention. Sodium 25 bisulfite (NaHSO_3) reacts readily with the 5,6-double bond of cytosine, but poorly with methylated cytosine. Cytosine reacts with the bisulfite ion to form a sulfonated cytosine reaction intermediate which is susceptible to deamination, giving rise to a sulfonated 30 uracil. The sulfonate group can be removed under alkaline conditions, resulting in the formation of uracil. Uracil is recognized as a thymine by Taq polymerase and therefore upon PCR, the resultant product contains

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cytosine only at the position where 5-methylcytosine occurs in the starting template DNA.

The primers used in the invention for amplification of the CpG-containing nucleic acid in the specimen, after bisulfite modification, specifically distinguish between untreated or unmodified DNA, methylated, and non-methylated DNA. MSP primers for the non-methylated DNA preferably have a T in the 3' CG pair to distinguish it from the C retained in methylated DNA, and the compliment is designed for the antisense primer. MSP primers usually contain relatively few Cs or Gs in the sequence since the Cs will be absent in the sense primer and the Gs absent in the antisense primer (C becomes modified to U (uracil) which is amplified as T (thymidine) in the amplification product).

The primers of the invention embrace oligonucleotides of sufficient length and appropriate sequence so as to provide specific initiation of polymerization on a significant number of nucleic acids in the polymorphic locus. Specifically, the term "primer" as used herein refers to a sequence comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and most preferably more than 8, which sequence is capable of initiating synthesis of a primer extension product, which is substantially complementary to a polymorphic locus strand.

Environmental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA polymerase, and a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate its strands before being used

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to prepare extension products. Preferably, the primer is an oligodeoxy ribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization.

5 The exact length of primer will depend on many factors, including temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 12-20 or more nucleotides, although it may contain fewer nucleotides.

10 Primers of the invention are designed to be "substantially" complementary to each strand of the genomic locus to be amplified and include the appropriate G or C nucleotides as discussed above. This means that the primers must be sufficiently complementary to
15 hybridize with their respective strands under conditions which allow the agent for polymerization to perform. In other words, the primers should have sufficient complementarity with the 5' and 3' flanking sequences to hybridize therewith and permit amplification of the
20 genomic locus. While exemplary primers are provided in SEQ ID NO:105-208, it is understood that any primer that hybridizes with the target sequences in SEQ ID NO: 1-104 is included in the invention and is useful in the method of the invention for detecting methylated nucleic acid,
25 as described.

Oligonucleotide primers of the invention are employed in the amplification process which is an enzymatic chain reaction that produces exponential quantities of target locus relative to the number of
30 reaction steps involved. Typically, one primer is complementary to the negative (-) strand of the locus and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed

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by extension with an enzyme, such as the large fragment of DNA Polymerase I (Klenow) and nucleotides, results in newly synthesized + and - strands containing the target locus sequence. Because these newly synthesized 5 sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (i.e., the target locus sequence) defined by the primer. The product of the chain reaction is a discrete nucleic acid duplex with 10 termini corresponding to the ends of the specific primers employed.

The oligonucleotide primers of the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods 15 or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al. (*Tetrahedron Letters*, 22:1859-1862, 1981). One method for synthesizing oligonucleotides on a 20 modified solid support is described in U.S. Patent No. 4,458,066.

Any nucleic acid specimen, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, provided it contains, or is suspected of 25 containing, the specific nucleic acid sequence containing the target locus (e.g., CpG). Thus, the process may employ, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. In the event that RNA is to be used as a 30 template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of nucleic acids may

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also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified, i.e., the target 5 locus, may be a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor 10 fraction of a complex mixture, such as contained in whole human DNA.

The nucleic acid-containing specimen used for detection of methylated CpG may be from any source including brain, colon, urogenital, hematopoietic, 15 thymus, testis, ovarian, uterine, prostate, breast, colon, lung and renal tissue and may be extracted by a variety of techniques such as that described by Maniatis, et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY, pp 280, 281, 1982).

20 If the extracted sample is impure (e.g., plasma, serum, stool, ejaculate, sputum, saliva, cerebrospinal fluid or blood or a sample embedded in parrafin), it may be treated before amplification with an amount of a reagent effective to open the cells, fluids, tissues, or 25 animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow amplification to occur much more readily.

30 Where the target nucleic acid sequence of the sample contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as the template. Strand separation can be effected either

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as a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished using various suitable denaturing conditions, including physical, chemical, or enzymatic

5 means, the word "denaturing" includes all such means.

One physical method of separating nucleic acid strands involves heating the nucleic acid until it is denatured. Typical heat denaturation may involve temperatures ranging from about 80° to 105°C for times ranging from

10 about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or by the enzyme RecA, which has helicase activity, and in the presence of riboATP, is known to denature DNA. The reaction conditions suitable for

15 strand separation of nucleic acids with helicases are described by Kuhn Hoffmann-Berling (CSH-Quantitative Biology, 43:63, 1978) and techniques for using RecA are reviewed in C. Radding (*Ann. Rev. Genetics*, 16:405-437, 1982).

20 When complementary strands of nucleic acid or acids are separated, regardless of whether the nucleic acid was originally double or single stranded, the separated strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This

25 synthesis is performed under conditions allowing hybridization of primers to templates to occur. Generally synthesis occurs in a buffered aqueous solution, preferably at a pH of 7-9, most preferably about 8. Preferably, a molar excess (for genomic nucleic

30 acid, usually about 10⁸:1 primer:template) of the two oligonucleotide primers is added to the buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not

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be known if the process of the invention is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, 5 however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve 10 the efficiency of the process.

The deoxyribonucleoside triphosphates dATP, dCTP, dGTP, and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 15 90°-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the solution is allowed to cool to room temperature, which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer 20 extension reaction (called herein "agent for polymerization"), and the reaction is allowed to occur under conditions known in the art. The agent for polymerization may also be added together with the other reagents if it is heat stable. This synthesis (or 25 amplification) reaction may occur at room temperature up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40°C. Most conveniently 30 the reaction occurs at room temperature.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes.

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Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, polymerase muteins, reverse transcriptase, 5 and other enzymes, including heat-stable enzymes (i.e., those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation). Suitable enzymes will facilitate combination of the nucleotides in the proper manner to 10 form the primer extension products which are complementary to each locus nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing 15 molecules of different lengths. There may be agents for polymerization, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above.

In nucleic acid hybridization reactions, the 20 conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid 25 type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

30 An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency

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conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, e.g., high stringency 5 conditions, or each of the conditions can be used, e.g., for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and 10 can be determined empirically.

Preferably, the method of amplifying is by PCR, as described herein and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be 15 employed as long as the methylated and non-methylated loci amplified by PCR using the primers of the invention is similarly amplified by the alternative means.

The amplified products are preferably identified as methylated or non-methylated by sequencing. Sequences 20 amplified by the methods of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction 25 (Saiki, et al., *Bio/Technology*, 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner, et al., *Proc. Natl. Acad. Sci. USA*, 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landegren, et al., *Science*, 241:1077, 1988), and the like. 30 Molecular techniques for DNA analysis have been reviewed (Landegren, et al., *Science*, 242:229-237, 1988).

Optionally, the methylation pattern of the nucleic acid can be confirmed by restriction enzyme digestion and

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Southern blot analysis. Examples of methylation sensitive restriction endonucleases which can be used to detect 5'CpG methylation include *Sma*I, *Sac*II, *Eag*I, *Msp*I, *Hpa*II, *Bst*UI and *Bss*HII, for example.

5 The invention provides a method for detecting a cell having a methylated CpG island or a cell proliferative disorder associated with methylated CpG in a tissue or biological fluid of a subject, comprising contacting a target cellular component suspected of
10 expressing a gene having a methylated CpG or having a CpG-associated disorder, with an agent which binds to the component. The target cell component can be nucleic acid, such as DNA or RNA, or protein. When the component is nucleic acid, the reagent is a nucleic acid probe or
15 PCR primer. When the cell component is protein, the reagent is an antibody probe. The probes can be detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator, or an
20 enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody, or will be able to ascertain such, using routine experimentation.

Actively transcribed genes generally contain fewer
25 methylated CGs than the average number in DNA. Hypermethylation can be detected by restriction endonuclease treatment and Southern blot analysis. Therefore, in a method of the invention, when the cellular component detected is DNA, restriction
30 endonuclease analysis is preferable to detect hypermethylation of the promoter for example. Any restriction endonuclease that includes CG as part of its recognition site and that is inhibited when the C is

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methylated, can be utilized. Preferably, the methylation sensitive restriction endonuclease is *BssHII*, *MspI*, or *HpaII*, used alone or in combination. Other methylation sensitive restriction endonucleases will be known to 5 those of skill in the art.

For purposes of the invention, an antibody or nucleic acid probe specific for a gene or gene product may be used to detect the presence of methylation either by detecting the level of polypeptide (using antibody) or 10 methylation of the polynucleotide (using nucleic acid probe) in biological fluids or tissues. For antibody based detection, the level of the polypeptide is compared with the level of polypeptide found in a corresponding "normal" tissue. Oligonucleotide primers based on any 15 coding sequence region of the promoter in the TIMP-2, estrogen receptor, GST-pi, calcitonin, HIC-1 or MLH1 sequence, for example, are useful for amplifying DNA, for example by PCR. These genes are merely listed as examples and are not meant to be limiting. Any specimen 20 containing a detectable amount of polynucleotide or antigen can be used. Preferably the subject is human.

The present invention provides the finding that TIMP-2 is methylated in cancer tissue as compared to normal tissue. For example, TIMP-2 was found to be 25 methylated in colon cancer tissue but not in normal colon tissue. The method for detecting a cell expressing a gene such as TIMP-2, or a cell proliferative disorder associated with methylation of CpG containing TIMP-2, or any gene including those described above, can be utilized 30 for detection of residual cancer or other malignancies in a subject in a state of clinical remission. Additionally, the method for detecting polypeptide in cells is useful for detecting a cell proliferative

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disorder by measuring the level of polypeptide in cells expressing the polypeptide, in a suspect tissue in comparison with the polypeptide expressed in normal cells or tissue. Using the method of the invention, expression 5 of any gene, such as TIMP-2, can be identified in a cell and the appropriate course of treatment can be employed (e.g., sense gene therapy or drug therapy). The expression pattern of the gene, e.g., TIMP-2, may vary with the stage of malignancy of a cell, therefore, a 10 sample such as breast or colon tissue can be screened with a panel of gene or gene product specific reagents (i.e., nucleic acid probes or antibodies) to detect gene expression, e.g., TIMP-2, and diagnose the stage of malignancy of the cell.

15 Monoclonal antibodies can be used in the method of the invention, for example, in immunoassays in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of 20 immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection 25 of the antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will 30 know, or can readily discern, other immunoassay formats without undue experimentation.

The term "immunometric assay" or "sandwich immunoassay", includes simultaneous sandwich, forward

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sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that antibodies according to the present invention will be useful in other 5 variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

Monoclonal antibodies can be bound to many 10 different carriers and used to detect the presence of TIMP-2. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of 15 the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

For purposes of the invention, TIMP-2 may be detected by the monoclonal antibodies when present in biological fluids and tissues. Any sample containing a detectable amount of TIMP-2 can be used. A sample can be a liquid such as ejaculate, urine, saliva, cerebrospinal 25 fluid, blood, serum and the like, or a solid or semi-solid such as tissues, feces, and the like, or, alternatively, a solid tissue such as those commonly used in histological diagnosis.

In performing the assays it may be desirable to 30 include certain "blockers" in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, proteases, or anti-heterophilic immunoglobulins

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to anti-TIMP-2 immunoglobulins present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive 5 or false negative results. The selection of "blockers" therefore may add substantially to the specificity of the assays described in the present invention.

In using a monoclonal antibody for the *in vivo* detection of antigen, the detectably labeled monoclonal 10 antibody is given in a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of the site having the TIMP-2 antigen for which 15 the monoclonal antibodies are specific.

The concentration of detectably labeled monoclonal antibody which is administered should be sufficient such that the binding to those cells having TIMP-2 is detectable compared to the background. Further, it is 20 desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

As a rule, the dosage of detectably labeled monoclonal antibody for *in vivo* diagnosis will vary 25 depending on such factors as age, sex, and extent of disease of the individual. The dosage of monoclonal antibody can vary from about 0.001 mg/m² to about 500 mg/m², preferably 0.1 mg/m² to about 200 mg/m², most preferably about 0.1 mg/m² to about 10 mg/m². Such 30 dosages may vary, for example, depending on whether multiple injections are given, tumor burden, and other factors known to those of skill in the art.

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For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given 5 type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious 10 radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

15 A monoclonal antibody useful in the method of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing 20 diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd , ^{55}Mn , ^{162}Dy , ^{52}Cr , and ^{56}Fe .

25 Monoclonal antibodies used in the method of the invention can be used to monitor the course of amelioration of TIMP-2 associated cell proliferative disorder. Thus, by measuring the increase or decrease in the number of cells expressing TIMP-2 or changes in TIMP- 30 2 present in various body fluids, it would be possible to determine whether a particular therapeutic regimen aimed at ameliorating the disorder is effective.

The term "modulate" envisions the suppression of methylation of TIMP-2 (e.g., promoter) or augmentation of TIMP-2 gene expression when TIMP-2 is under-expressed. When a cell proliferative disorder is associated with 5 TIMP-2 expression, such methylation suppressive reagents as 5-azacytadine can be introduced to a cell. Alternatively, when a cell proliferative disorder is associated with under-expression of TIMP-2 polypeptide, a sense polynucleotide sequence (the DNA coding strand) 10 encoding the promoter region or the promoter operably linked to the structural gene, or TIMP-2 polypeptide can be introduced into the cell.

The present invention also provides gene therapy for the treatment of cell proliferative disorders which 15 are mediated by TIMP-2. Such therapy would achieve its therapeutic effect by introduction of the appropriate TIMP-2 polynucleotide which contains either a normal TIMP-2 promoter region alone or in combination with a TIMP-2 structural gene (sense), into cells of subjects 20 having the proliferative disorder. Alternatively, the TIMP-2 structural gene could be introduced operably linked to a heterologous promoter. Delivery of sense TIMP-2 promoter polynucleotide constructs can be achieved 25 using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system.

The promoter polynucleotide sequences used in the method of the invention may be the native, unmethylated sequence or, alternatively, may be a sequence in which a nonmethylatable analog is substituted within the 30 sequence. Preferably, the analog is a nonmethylatable analog of cytidine, such as 5-azacytadine. Other analogs will be known to those of skill in the art. Alternatively, such nonmethylatable analogs could be

administered to a subject as drug therapy, alone or simultaneously with a sense promoter for TIMP-2 or a sense promoter operably linked with the structural gene for the corresponding gene (e.g., TIMP-2 promoter with 5 TIMP-2 structural gene).

The invention also relates to a medicament or pharmaceutical composition comprising a TIMP-2 promoter polynucleotide or a TIMP-2 promoter polynucleotide operably linked to the TIMP-2 structural gene, 10 respectively, in a pharmaceutically acceptable excipient or medium wherein the medicament is used for therapy of TIMP-2 associated cell proliferative disorders.

The invention also provides the use of MSP for *in situ* methylation analysis. For example, MSP can be used 15 to detect methylation of DNA in the nucleus of an intact cell. A tissue section, a cell or population of cells is placed or immobilized on a solid support (e.g., a slide) and MSP primers used directly on the cell for amplification of the appropriate sequences. The primers 20 are typically detectably labeled with a reporter means, e.g., fluorescent label. Alternatively, a probe that detects or hybridizes with the MSP amplified sequences is used to detect amplification of methylated sequences. *In situ* methylation analysis using MSP is useful, for 25 example, in detecting nucleic acid having a mutant nucleotide sequence associated with a primary tumor in the adjacent histopathologic surgical margins and more distant tissues, such as regional lymph nodes, which are apparently "normal" when examined by standard 30 histological techniques. Using MSP, it is possible to detect target nucleic acids from cells previously associated with a large number of disease states which are present in tissue that appears normal. MSP *in situ*

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can be used as an adjunct to cytopathology, to screen high-risk populations and to monitor high risk patients undergoing chemoprevention or chemotherapy.

Exemplary target polynucleotide sequences to which 5 the primer of the invention hybridizes have a sequence as listed below.

		<u>SEQ ID NO.</u>
	Wild type p16	1
10	5'-GCGGTCCGCCAACCTCTG-3'; 5'-CCACGGCCGGCCCG- 3';	2
	Methylated p16-1*	3
	5'-GCGATCCGCCAACCTCTAATAA-3'; 5'-TTACGGTCGGTTCGGGTC-3';	4
	Unmethylated p16-1	5
	5'-ACAATCCACCCACCCCTCTAATAA-3'; 5'-TTATGGTTGGTTGGGTTG-3';	6
15	Methylated p16-2	7
	5'-GCGATCCGCCAACCTCTAATAA-3'; 5'-CGGTCGGAGGTCGATTAGGTGG-3';	8
	Unmethylated p16-2	9
	5'-ACAATCCACCCACCCCTCTAATAA-3'; 5'-TGGTTGGAGGTTGATTAGGTGG-3';	10
20	Wild type p15	11
	5'-TCTGGCCGCAGGGTGCG-3'; 5'-CCGGCCGCTCGGCCACT-3';	12
	Methylated p15	13
	5'-AACCGCAAAATACGAACGC-3'; 5'-TCGGTCGTTGGTTATTGTACG-3';	14
	Unmethylated p15	15
	5'-AACCACAAAATACAAACACATCACA-3'; 5'-TTGGTTGGTTATTGTATGG-3';	16
25	Methylated VHL	17
	5'-GCGTACGCAAAAAATCCTCCA-3'; 5'-TTCGCGGCGTTCGGTT-3';	18
	Unmethylated VHL	19
	5'-ACATACACAAAAAATCCTCCAAC-3'; 5'-TTTGTGGTGGTTGGTTGGG-3';	20

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	Methylated		
	E-cadherin	5'-ACGCGATAACCCTCTAACCTAA-3';	21
		5'-GTCGGTAGGTGAATTTAGTTA-3';	22
	Unmethylated		
5	E-cadherin	5'-ACAATAACCCTCTAACCTAAAATTA-3';	23
		5'-TGTGTTGTTGATTGGTTGTG-3';	24
	Methylated Androgen		
	Receptor	5'-GCGACCTCTAAATACCTAAAACCC-3';	25
		5'-CGTAGAGGTTTATAGGTTATTGGA-3';	26
10	Unmethylated		
	Androgen Receptor	5'-ACAACCTCTAAATACCTAAAACCC-3';	27
		5'-TGTAGAGGTTTATAGGTTATTGGT-3';	28
	Methylated Estrogen		
15	Receptor	5'-GACGAACTTACTACTATCCAAATACAC-3';	29
		5'-TTTACGGTTAGATCGGTTTTTACG-3';	30
	Unmethylated		
	Estrogen Receptor	5'-AACAAACTTACTACTATCCAAATACACC-3';	31
		5'-TGGTTAGATTGGTTTTTATGG-3';	32
20	Methylated MDGI	5'-GCCCGACTCCGAAATAAA-3';	33
		5'-CGTCGTCGGAGTTTGTACGTT-3';	34
	Unmethylated MDGI		
		5'-ACCCCCAACTCCAAAATAAAAAA-3';	35
		5'-TGTTGTTGGAGTTTGTATGTT-3';	36
	Methylated GSTp		
25		5'-GACGACCGCTACACCCCGAA-3';	37
		5'-CGTCGTGATTTAGTATTGGGGC-3';	38
	Unmethylated GSTp		
		5'-ACAACCACTACACCCCAAACATC-3';	39
		5'-TGTTGTGATTTAGTATTGGGGTGG-3';	40
	Methylated		
30	Calcitonin	5'-GCCAACGACTACTCTTATTCCCG-3';	41
		5'-CGTCGTCGTTTATAGGGTTTG-3';	42

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	Unmethylated		
	Calcitonin	5'-ACCAACAACTACTCTTATTCCCACC-3';	43
		5'-TGTTGTTTTTATAGGGTTTGG-3';	44
	Methylated HIC-1	5'-GACGCACAACCGACTACGAC-3';	45
5		5'-CGGCGTTAGGGCGGGTATC-3';	46
	Unmethylated HIC-1	5'-AACACACAACCAACTACAACCC-3';	47
		5'-TGGTGTTAGGGTGGGTATTGTG-3';	48
	Methylated		
	Endothelin	5'-GCGTAACCAAAAAAAATAATAATAC-3';	49
10		5'-CGCGTTGGTGAGTTATGA-3';	50
	Unmethylated		
	Endothelin	5'-ACATAACCAAAAAAAATAATAATACAA-3';	51
		5'-TGTGTTGGTGAGTTATGAGTGTAAAG-3';	52
15	Methylated TIMP-2	5'-GACCGCGCTACCTTCTACGAATAT-3';	53
		5'-CGCGGGAGGGGTTCGTT-3';	54
	Unmethylated TIMP-2	5'-AACCAACACTACCTTCTACAAATATATTACTA-3';	55
		5'-TGTGGGAGGGGTTGTTTG-3';	56
	Methylated MLH1-a	5'-GCGACCCTAATAAAACGTCTACGT-3';	57
20		5'-CGCGGGTAGTTACGATGAGG-3';	58
	Unmethylated MLH1-a	5'-ACAACCCTAATAAAACATCTACATCAAA-3';	59
		5'-TGTGGGTAGTTATGATGAGGTGGT-3';	60
	Methylated MLH1-b	5'-GAACGACATTTAACGCCAAAA-3';	61
25		5'-CGGCGGGGAAGTTATTTA-3';	62
	Unmethylated MLH1-b	5'-AAACAACATTTAACACCAAAAAACC-3';	63
		5'-TGGTGGGGGAAGTTATTTAGTGG-3';	64
	Methylated MSH2	5'-GAACGACGTCCGACCACGA-3';	65
		5'-CGGTGTAGTCGAAGGAGACGTTG-3';	66
30	Unmethylated MSH2	5'-AAACAACATCCAACCACAACC-3';	67
		5'-TGGTGTAGTTGAAGGAGATGTTAGTTG-3';	68

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Methylated GFAP	5'-GATACCCGAATACCCCTAACAAAC-3'; 5'-CGTCGTTTACGTTTTAGGG-3';	69 70
Unmethylated GFAP	5'-AATACCCAAATACCCCTAACAAACA-3' 5'-TGTGTTTATGTTTTAGGGGA-3'	71 72
5 Methylated TGFb1	5'-GCGAACTACCAAACGAACCCA-3'; 5'-CGCGCGGTTAGGGAGGG-3';	73 74
Unmethylated TGFb1	5'-ACAAACTACCAAACAAACCCAACC-3'; 5'-TGTGGTGGTTAGGGAGGTGGG-3';	75 76
10 Methylated TGFb2	5'-GCGCGAAAATATCGTCG-3'; 5'-CGCGTTCGTCGGTT-3';	77 78
Unmethylated TGFb2	5'-ACACAAAAATATCATCACTCCATAC-3'; 5'-TGTGTTTGTGGTTTTAGGT-3';	79 80
15 Methylated p130	5'-GACGCTAACCGCCTACAAACA-3'; 5'-CGGTCGTTAGGGGTGCGT-3';	81 82
Unmethylated p130	5'-AACACTAACCAACCTACAAACACCCA-3'; 5'-TGGTTGTTAGGGGTGTTATGTT-3';	83 84
20 Methylated BRCA2	5'-GACTCCGCCTCTACCGC-3'; 5'-CGGTTTTGTTAGTTATTCG-3';	85 86
Unmethylated BRCA2	5'-AACTCCACCTCTACCACCTAAT-3'; 5'-TGGTTTTGTTAGTTATTTGG-3';	87 88
25 Methylated O6-MGMT	5'-GCGCGAAAACGAAACCGA-3'; 5'-CGCGTTCGGATATGTTGGG-3';	89 90
Unmethylated O6-MGMT	5'-ACACAAAAACAAAACCAAAACAC-3'; 5'-TGTGTTGGATATGTTGGGA-3';	91 92
Methylated NF1	5'-AACGTCCCTAACGCCGTAA-3'; 5'-CGTATGCGCGGTAGGTCGTT-3';	93 94

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Unmethylated NF1	5'-AAACATCCCTAACACCCATAAAACTC-3'; 5'-TGTATGTGTGGTAGGTTTTTTTT-3';	95 96
Methylated NF2	5'-GCGAAACTCAAACCCGAAAC-3'; 5'-CGTTTATCGTGAGGATCGTTATTAT-3';	97 98
5		
Unmethylated NF2	5'-ACAAAACCTCAAACCCAAAACCC-3'; 5'-TGTATTGTGAGGATTGTTATTATGG-3';	99 100
Methylated TSG101	5'-GCTACTAAACTACCCCAAACCGTC-3'; 5'-CGGTCGTTATGGCGGTGTC-3';	101 102
10		
Unmethylated TSG101	5'-ACTACTAAACTACCCCAAACCATCC-3'; 5'-TGGTTGTTATGGTGGTGGAG-3';	103 104

Exemplary primer pairs included in the invention
that hybridize to the above sequences include:

15		<u>SEQ ID NO:</u>
	5'-CAGAGGGTGGGGCGGACCGC-3' and 5'-CGGGCCGCGGCCGTGG-3';	105 106
	5'-TTATTAGAGGGTGGGGCGGATCGC-3' and 5'-GACCCCGAACCGCGACCGTAA-3';	107 108
20	5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCCCAAACCACAACCATAA-3';	109 110
	5'-TTATTAGAGGGTGGGGCGGATCGC-3' and 5'-CCACCTAAATCGACCTCCGACCG-3';	111 112
	5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CCACCTAAATCAACCTCCAACCA-3';	113 114
25	5'-CGCACCTGCGGCCAGA-3' and 5'-AGTGGCCGAGCGGCCGG-3';	115 116
	5'-GCGTTCGTATTTGCGGTT-3' and 5'-CGTACAATAACCGAACGACCGA-3';	117 118

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	5'-TGTGATGTGTTGTATTTGTGGTT-3' and	119
	5'-CCATACAATAACCAAACAAACCAA-3' ;	120
	5'-TGGAGGATTTTGTGGTACGC-3' and	121
	5'-GAACCGAACGCCCGCAA-3' ;	122
5	5'-GTTGGAGGGATTTTGTGTATGT-3' and	123
	5'-CCCAAACCAAACACCACAAA-3' ;	124
	5'-TTAGGTTAGAGGGTTATCGCGT-3' and	125
	5'-TAACTAAAAATTCACCTACCGAC-3' ;	126
	5'-TAATTTAGGTAGAGGGTTATTGT-3' and	127
10	5'-CACAACCAATCAACAAACACA-3'	128
	5'GGGTTTTAGGTATTTAGAGGTTCGC-3' and	129
	5'ACCAAATAACCTATAAAACCTCTACG-3'	130
15	5'GGGTTTTAGGTATTTAGAGGTGT-3' and	131
	5'ACCAAATAACCTATAAAACCTCTAC-3'	132
	5'GTGTATTTGGATAGTAGTAAGTTCGTC-3' and	133
	5'CGTAAAAAAACCGATCTAACCGTAAA-3'	134
	5'GGTGTATTTGGATAGTAGTAAGTTGTT-3' and	135
20	5'CCATAAAAAAAACCAATCTAACCA-3'	136
	5'TTTATTCGGGAGTCGGGGC-3' and	137
	5'AACGTACAAAAACTCCGACGACG-3'	138
	5'TTTTTATTTGGAGTTGGGGT-3' and	139
25	5'AAACATACAAAAACTCCAACAAACA-3'	140
	5'TTCGGGGTGTAGCGGTGTC-3' and	141
	5'GCCCAATACTAAATCACGACG-3'	142
	5'GATTTGGGTGTAGTGGTTGTT-3' and	143
30	5'CCACCCCAATACTAAAATCACAAACA-3'	144

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	5' CGGGAATAAGAGTAGTCGTTGGC-3' and	145
	5' CAAAACCTATAAAAACGACGACG-3'	146
	5' GGTGGGAATAAGAGTAGTTGTTGGT-3' and	147
5	5' CCAAAACCCCTATAAAAACAACAAACA-3'	148
	5' GTCGTAGTCGGTTGTGCGTC-3' and	149
	5' GATACCCGCCCTAACGCCG-3'	150
	5' GGGTTGTAGTTGGTTGTGTGTT-3' and	151
10	5' CACAATACCCACCCTAACACCA-3'	152
	5' GTATATTATTTATTTTTGGTTACGC-3' and	153
	5' TCATAACTGCCAACGCG-3'	154
	5' TTGTATATTATTTATTTTTGGTTATGT-3' and	155
15	5' CTTAACACTCATAACTCACCAACACA-3'	156
	5' ATATTCGTAGAAGGTAGCGCGGTC-3' and	157
	5' AACGAACCCCTCCCGCG-3'	158
	5' TAGTAAATATTTGTAGAAGGTAGTGTGGTT-3' and	159
20	5' CAAACAAACCCCTCCCACA-3'	160
	5' ACGTAGACGTTTATTAGGGTCGC-3' and	161
	5' CCTCATCGTAACTACCCCGCG-3'	162
	5' TTTTGATGTAGATGTTTATTAGGGTTGT-3' and	163
25	5' ACCACCTCATCATAACTACCCACA-3'	164
	5' TTTTGGCGTTAAATGTCGTT-3' and	165
	5' TAAATAACTCCCCCGCCG-3'	166
	5' GGTTTTTGGTGTAAATGTTGTT-3' and	167
30	5' CCACTAAATAACTCCCCCACCA-3'	168
	5' TCGTGGTCGGACGTCGTT-3' and	169
	5' CAACGTCTCCTTCGACTACACCG-3'	170

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	5' GGTTGTTGTGGTGGATGTTGTT-3' and	171
	5' CAACTACAAACATCTCCTTCAACTACACCA-3'	172
	5' GTTGTAGGGGTATCGGGTATC-3' and	173
	5' CCCTAAAAAAACGTAAAACGACG-3'	174
5	5' TGTTGTAGGGTATTTGGGTATT-3' and	175
	5' TCCCCTAAAAAAACATAAAAACAACA-3'	176
	5' TGGGTTCGTTGGTAGTCGC-3' and	177
	5' CCTCCCTAACCGCCGCG-3'	178
10	5' GGTTGGGTTTGGTAGTTGT-3' and	179
	5' CCCACCTCCCTAACCAACCACACA-3'	180
	5' CGACGATATTCGCGC-3' and	181
	5' AACCGACGAAACGCG-3'	182
15	5' GTATGGAGTGATGATATTTGTGT-3' and	183
	5' ACCTAAAAACCAACAAAACACA-3'	184
	5' TGTTGTAGGCGGTTAGCGTC-3' and	185
	5' ACGCACCCCTAAACGACCG-3'	186
20	5' TGGGTGTTGTAGGTGGTTAGTGT	187
	5' AACATAACACACCCCTAAACAAACCA-3'	188
	5' GCGGTAGAGGCAGGAGTC-3' and	189
	5' CGAAATAAACTAACAAAAACCG-3'	190
25	5' ATTAGGTGGTAGAGGTGGAGTT-3' and	191
	5' CCAAAATAAACTAACAAAAACCA-3'	192
	5' TCGTTTCGTTTGCAGC-3' and	193
	5' CCCAACATATCCGAAACGCG-3'	194
30	5' GTGTTTGGTTTGTGT-3' and	195
	5' TCCCAACATATCCAAAACACA-3'	196

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	5' TTACGGCGTTGAGGGACGTTC-3' and	197
	5' AAACGACCTACCGCGCATACG-3'	198
	5' GAGTTTATGGTGTGAGGGATGTTT-3' and	199
5	5'AAAAAAAAAAACAACCTACCAACACATACA-3'	200
	5' GTTCGGGTTGAGTTTCGC-3' and	201
	5' ATAATAACGATCCTCACGATAAACG-3'	202
10	5' GGGTTTGGGTTGAGTTTGT-3' and	203
	5' CCATAATAACAATCCTCACAAATAACA-3'	204
	5' GACGGTTGGGGTAGTTAGTGC-3' and	205
	5' GACACCGCCATAACGACCG-3'	206
15	5' GGATGGTTGGGGTAGTTAGTAGT-3' and	207
	5' CTCCAACACCACCATACAAACCA-3'	208

*Also included are modifications of the above sequences, including SEQ ID NO:107 having the sequence TCAC at the 5' end; SEQ ID NO:107 having the sequence CC added at the 5' end; SEQ ID NO:108 having the sequence 5'-TTATTAGAGGGTGGGGCGGATCGC-3'; SEQ ID NO:109 having the sequence 5'-GACCCCGAACCGCGACCGTAA-3'; SEQ ID NO:110 having the sequence TGG added at the 5' end; and SEQ ID NO:111 having the sequence TACC added at the 5' end. All of these modified primers anneal at 65°C.

25 Typically, the CpG-containing nucleic acid is in the region of the promoter of a structural gene. For example, the promoter region of tumor suppressor genes have been identified as containing methylated CpG island. The promoter region of tumor suppressor genes, including 30 p16, p15, VHL and E-cadherin, are typically the sequence amplified by PCR in the method of the invention. Other genes that have been shown by MSP as containing methylated CpG neoplastic versus normal tissue include estrogen receptor, MDGI, GST-pi, calcitonin, HIC-1, 35 endothelin B receptor, TIMP-2, 06-MGMT, and MLH1. Genes

that were found by MSP to be methylated also include the androgen receptor (e.g., methylated as X chromosome inactivation), GFAP (methylated in some glioma cell lines but also in normal tissue), and MSH2. Other genes in 5 which MSP primer were shown to distinguish between normal unmethylated and methylated DNA include TGF- β 1, TGF- β 2, p130, BRCA2, NF1, NF2, and TSG101.

Detection and identification of methylated CpG-containing nucleic acid in the specimen may be indicative 10 of a cell proliferative disorder or neoplasia. Such disorders include but are not limited to low grade astrocytoma, anaplastic astrocytoma, glioblastoma, medulloblastoma, colon cancer, lung cancer, renal cancer, leukemia, breast cancer, prostate cancer, endometrial 15 cancer and neuroblastoma. Identification of methylated CpG status is also useful for detection and diagnosis of genomic imprinting, fragile X syndrome and X-chromosome inactivation.

Using the method of the invention, the TIMP-2 gene 20 was identified as associated with or methylated in neoplastic versus normal tissues.

The method of the invention now provides the basis for a kit useful for the detection of a methylated CpG-containing nucleic acid. The kit includes a carrier means 25 being compartmentalized to receive in close confinement therein one or more containers. For example, a first container contains a reagent which modifies unmethylated cytosine, such as sodium bisulfite. A second container contains primers for amplification of the CpG-containing 30 nucleic acid, for example, primers listed above as SEQ ID NO:105-208.

The invention also provides a kit for the detection of a methylated CpG-containing nucleic acid,

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wherein the kit includes: a) a reagent that modifies unmethylated cytosine nucleotides; b) control nucleic acid; c) primers for the amplification of unmethylated CpG-containing nucleic acid; d) primers for the 5 amplification of methylated CpG-containing nucleic acid; and e) primers for the amplification of control nucleic acid. The kit may further include nucleic acid amplification buffer. Preferably, the reagent that modifies unmethylated cytosine is bisulfite.

10 The kit of the invention is intended to provide the reagents necessary to perform chemical modification and PCR amplification of DNA samples to determine their methylation status. The primer sets included in the kit include a set that anneals to unmethylated DNA that has 15 undergone a chemical modification; a set that anneals to methylated DNA that has undergone a chemical modification; and a primer set that serves as a control for the efficiency of chemical modification. The control primer set should anneal to any DNA (unmethylated or 20 methylated) that has not undergone chemical methylation. In the case of incomplete chemical modification (up to about 50%), data interpretation can still proceed.

The above disclosure generally describes the present invention. A more complete understanding can be 25 obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

Example 1

30 **DNA and Cell Lines.** Genomic DNA was obtained from cell lines, primary tumors and normal tissue as described (Merlo, et al., *Nature Medicine*, 1:686, 1995; Herman, et

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al., *Cancer Research*, 56:722, 1996; Graff, et al., *Cancer Research*, 55:5195, 1995). The renal carcinoma cell line was kindly provided by Dr. Michael Lehrman of the National Cancer Institute, Bethesda, MD.

5 **Bisulfite Modification.** 1 μ g of DNA in a volume of 50 μ l was denatured by NaOH (final 0.2M) for 10 minutes at 37 °C. For samples with nanogram quantities of human DNA, 1 μ g of salmon sperm DNA (Sigma) was added as carrier prior to modification. 30 μ L of 10mM hydroquinone 10 (Sigma) and 520 μ L of 3 M sodium bisulfite (Sigma) pH5, both freshly prepared, were added, mixed, and samples were incubated under mineral oil at 50 °C for 16 hours. Modified DNA was purified using the Wizard™ DNA purification resin according to the manufacturer 15 (Promega), and eluted into 50 μ L of water. Modification was completed by NaOH (final 0.3M) treatment for 5 minutes at room temperature, followed by ethanol precipitation.

20 **Genomic Sequencing.** Genomic sequencing of bisulfite modified DNA was accomplished using the solid-phase DNA sequencing approach (Myohanen, et al., *DNA Seq.*, 5:1, 1994). 100 ng of bisulfite modified DNA was amplified with *p16* gene specific primer 5'-TTTTAGAGGATTTGAGGGATAGG-3' (sense) (SEQ ID NO:209) and 25 5'-CTACCTAATTCCAATTCCCCCTACA-3' (anti-sense) (SEQ ID NO:210). PCR conditions were as follows: 96 °C for 3 minutes, 80 °C for 3 minutes, 1 U of *Taq* polymerase (BRL) was added, followed by 35 cycles of 96 °C for 20 seconds, 56 °C for 20 seconds, 72 °C for 90 seconds, followed by 5 30 minutes at 72 °C. The PCR mixture contained 1X buffer (BRL) with 1.5mM MgCl₂, 20 pmols of each primer and 0.2 mM dNTPs. To obtain products for sequencing, a second round

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of PCR was performed with 5 pmols of nested primers. In this reaction, the sense primer,

5'-GTTTCCCAGTCACGACAGTATTAGGAGGAAGAAAGAGGAG-3' (SEQ ID NO:211), contains M13-40 sequence (underlined)

5 introduced as a site to initiate sequencing, and the anti-sense primer

5'-TCCAATTCCCCTACAACTTC-3" (SEQ ID NO:212) is biotinylated to facilitate purification of the product prior to sequencing. PCR was performed as above, for 32 10 cycles with 2.5 mM MgCl₂. All primers for genomic sequencing were designed to avoid any CpGs in the sequence. Biotinylated PCR products were purified using streptavidin coated magnetic beads (Dynal AB, Norway), and sequencing reactions performed with Sequenase™ and 15 M13-40 sequencing primer under conditions specified by the manufacturer (USB).

PCR Amplification. Primer pairs described in Table 1 were purchased from Life Technologies. The PCR mixture contained 1X PCR buffer (16.6 mM ammonium sulfate, 67mM 20 TRIS pH 8.8, 6.7 mM MgCl₂, and 10 mM β-mercaptoethanol), dNTPs (each at 1.25mM), primers (300 ng/reaction each), and bisulfite-modified DNA (~50ng) or unmodified DNA (50-100ng) in a final volume of 50 μL. PCR specific for unmodified DNA also included 5% dimethylsulfoxide. 25 Reactions were hot started at 95 °C for 5 minutes prior to the addition of 1.25 units of Taq polymerase (BRL). Amplification was carried out on a Hybaid OmniGene temperature cycler for 35 cycles (30 seconds at 95°C, 30 seconds at the annealing temperature listed in Table 1, 30 and 30 seconds at 72 °C), followed by a final 4 minute extension at 72 °C. Controls without DNA were performed for each set of PCR reactions. 10 μL of each PCR reaction was directly loaded onto non-denaturing 6-8%

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polyacrylamide gels, stained with ethidium bromide, and directly visualized under UV illumination.

Restriction Analysis. 10 μ L of the 50 μ L PCR reaction was digested with 10 units of *Bst*UI (New England 5 Biolabs) for 4 hours according to conditions specified by the manufacturer. Restriction digests were ethanol precipitated prior to gel analysis.

Example 2

An initial study was required to validate the 10 strategy for MSP for providing assessment of the methylation status of CpG islands. The p16 tumor suppressor (Merlo, et al., *supra*; Herman, et al., *Cancer Research*, 55:4525, 1995; Gonzalez-Zulueta, et al., *Cancer Res.*, 55:4531, 1995,27) which has been documented to have 15 hypermethylation of a 5' CpG island is associated with complete loss of gene expression in many cancer types, was used as an exemplary gene to determine whether the density of methylation, in key regions to be tested, was great enough to facilitate the primer design disclosed 20 herein. Other than for CpG sites located in recognition sequences for methylation-sensitive enzymes, the density of methylation and its correlation to transcriptional silencing had not yet been established. The genomic sequencing technique was therefore employed to explore 25 this relationship.

Figure 1 shows genomic sequencing of p16. The sequence shown has the most 5' region at the bottom of the gel, beginning at +175 in relation to a major transcriptional start site (Hara, et al., *Mol. Cell Biol.*, 16:859, 1996). All cytosines in the unmethylated 30 cell line H249 have been converted to thymidine, while all C's in CpG dinucleotides in the methylated cell H157

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remains as C, indicating methylation.] enclosed a *Bst*UI site which is at -59 in relation to the transnational start site in Genbank sequence U12818 (Hussussian, et al., *Nat. Genet.*, 8:15, 1994), but which is incorrectly 5 identified as CGCA in sequence X94154 (Hara, et al., *supra*). This CGCG site represents the 3' location of the sense primer used for *p16* MSP.

As has been found for other CpG islands examined in this manner (Myohanen, et al., *supra*; Park, et al., 10 *Mol. Cell Biol.*, 14:7975, 1994; Reeben, et al., *Gene*, 157:325, 1995), the CpG island of *p16* was completely unmethylated in those cell lines and normal tissues previously found to be unmethylated by Southern analysis (Fig. 1) (Merlo, et al., *supra*; Herman, et al., *supra*). 15 However, it was extensively methylated in cancer cell lines shown to be methylated by Southern analysis (Fig. 1). In fact, all cytosines within CpG dinucleotides in this region were completely methylated in the cancers lacking *p16* transcription. This marked difference in 20 sequence following bisulfite treatment suggested that the method of the invention for specific amplification of either methylated or unmethylated alleles was useful for identification of methylation patterns in a DNA sample.

Primers were designed to discriminate between 25 methylated and unmethylated alleles following bisulfite treatment, and to discriminate between DNA modified by bisulfite and that which had not been modified. To accomplish this, primer sequences were chosen for regions containing frequent cytosines (to distinguish unmodified 30 from modified DNA), and CpG pairs near the 3' end of the primers (to provide maximal discrimination in the PCR reaction between methylated and unmethylated DNA). Since the two strands of DNA are no longer complementary after

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bisulfite treatment, primers can be designed for either modified strand. For convenience, primers were designed for the sense strand. The fragment of DNA to be amplified was intentionally small, to allow the assessment of 5 methylation patterns in a limited region and to facilitate the application of this technique to samples, such as paraffin blocks, where amplification of larger fragments is not possible. In Table 1, primer sequences are shown for all genes tested, emphasizing the 10 differences in sequence between the three types of DNA which are exploited for the specificity of MSP. The multiple mismatches in these primers which are specific for these different types of DNA suggest that each primer set should provide amplification only from the intended 15 template.

The primers designed for *p16* were tested with DNA from cancer cell lines and normal tissues for which the methylation status had previously been defined by Southern analysis (Merlo, et al., *supra*; Herman, et al., 20 *supra*).

Figure 2, panels A-D, show polyacrylamide gels with the Methylation Specific PCR products of *p16*. Primer sets used for amplification are designated as unmethylated (U), methylated (M), or unmodified/wild-type 25 (W).* designates the molecular weight marker pBR322-*MspI* digest. Panel A shows amplification of bisulfite-treated DNA from cancer cell lines and normal lymphocytes, and untreated DNA (from cell line H249). Panel B shows mixing of various amount of H157 DNA with 1 μ g of H249 DNA prior 30 to bisulfite treatment to assess the detection sensitivity of MSP for methylated alleles. Modified DNA from a primary lung cancer sample and normal lung are also shown. Panel C shows amplification with the *p16-U2*

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(U) primers, and p16-M2 (M) described in Table 1. Panel D shows the amplified *p16* products of panel C restricted with *BstUI*(+) or not restricted (-).

In all cases, the primer set used confirmed the 5 methylation status determined by Southern analysis. For example, lung cancer cell lines U1752 and H157, as well other cell lines methylated at *p16*, amplified only with the methylated primers (Fig. 2, panel A). DNA from normal tissues (lymphocytes, lung, kidney, breast, and colon) 10 and the unmethylated lung cancer cell lines H209 and H249, amplified only with unmethylated primers (examples in Fig 2, panel A). PCR with these primers could be performed with or without 5% DMSO. DNA not treated with bisulfite (unmodified) failed to amplify with either set 15 of methylated or unmethylated specific primers, but readily amplified with primers specific for the sequence prior to modification (Fig. 2, panel A). DNA from the cell line H157 after bisulfite treatment also produced a weaker amplification with unmodified primers, suggesting 20 an incomplete bisulfite reaction. However, this unmodified DNA, unlike partially restricted DNA in previous PCR assays relying on methylation sensitive restriction enzymes, is not recognized by the primers specific for methylated DNA. It therefore does not 25 provide a false positive result or interfere with the ability to distinguish methylated from unmethylated alleles.

The sensitivity of MSP for detection of methylated *p16* alleles was assessed. DNA from methylated cell lines 30 was mixed with unmethylated DNA prior to bisulfite treatment. 0.1% of methylated DNA (approximately 50 pg) was consistently detected in an otherwise unmethylated sample (Fig. 2, panel B). The sensitivity limit for the

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amount of input DNA was determined to be as little as 1 ng of human DNA, mixed with salmon sperm DNA as a carrier detectable by MSP.

Fresh human tumor samples often contain normal and 5 tumor tissue, making the detection of changes specific for the tumor difficult. However, the sensitivity of MSP suggests it would be useful for primary tumors as well, allowing for detection of aberrantly methylated alleles even if they contribute relatively little to the overall 10 DNA in a sample. In each case, while normal tissues were completely unmethylated, tumors determined to be methylated at *p16* by Southern analysis also contained methylated DNA detected by MSP, in addition to some 15 unmethylated alleles (examples in Fig. 2, panel B). DNA from paraffin-embedded tumors was also used, and allowed the detection of methylated and unmethylated alleles in these samples (Fig. 2, panel B). To confirm that these results were not unique to this primer set, a second 20 downstream primer for *p16* was used which would amplify a slightly larger fragment (Table 1). This second set of primers reproduced the results described above (Fig 2, panel C), confirming the methylation status defined by 25 Southern blot analysis.

To further verify the specificity of the primers 25 for the methylated alleles and to check specific cytosines for methylation within the region amplified, the differences in sequence between methylated/modified DNA and unmethylated/modified DNA were utilized. Specifically, the *BstUI* recognition site, CGCG, will 30 remain CGCG if both C's are methylated after bisulfite treatment and amplification, but will become TGTG if unmethylated. Digestion of the amplified products with *BstUI* distinguishes these two products. Restriction of

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p16 amplified products illustrates this. Only unmodified products and methylated/modified products, both of which retain the CGCG site, were cleaved by *Bst*UI, while products amplified with unmethylated/modified primers 5 failed to be cleaved (Fig. 2, panel D).

The primer sets discussed above were designed to discriminate heavily methylated CpG islands from unmethylated alleles. To do this, both the upper (sense) and lower (antisense) primers contained CpG sites which 10 could produce methylation-dependent sequence differences after bisulfite treatment. MSP might be employed to examine more regional aspects of CpG island methylation. To examine this, methylation-dependent differences in the sequence of just one primer was tested to determine 15 whether it would still allow discrimination between unmethylated and methylated *p16* alleles. The antisense primer used for genomic sequencing, 5'-
CTACCTAATTCCAATTCCCCTACA-3' (SEQ ID NO:213), was also used as the antisense primer, since the region recognized 20 by the primer contains no CpG sites, and was paired with either a methylated or unmethylated sense primer (Table 1). Amplification of the 313 bp PCR product only occurred with the unmethylated sense primer in H209 and H249 (unmethylated by Southern) and the methylated sense 25 primer in H157 and U1752 (methylated by Southern), indicating that methylation of CpG sites within a defined region can be recognized by specific primers and distinguish between methylated and unmethylated alleles (Fig. 2, panel E). Panel E shows results of testing for 30 regional methylation of CpG islands with MSP, using sense primers *p16-U2* (U) and *p16-M2* (M), which are methylation specific, and an antisense primer which is not methylation specific.

Example 3

The above experiments with p16 were extended to include 3 other genes transcriptionally silenced in human cancers by aberrant hypermethylation of 5' CpG islands.

5 Figure 3, panels A-E, show polyacrylamide gels of MSP products from analysis of several genes. Primer sets used for amplification are not designated as unmethylated (U), methylated (M), or unmodified/wild-type (W). * designates the molecular weight marker pBR322-MspI digest 10 and ** designates the 123bp molecular weight marker. All DNA samples were bisulfite treated except those designated untreated. Panel A shows the results from MSP for *p15*. Panel B shows the *p15* products restricted with *BstUI* (+) or not restricted (-). Panel C shows the 15 products of MSP for VHL. Panel D shows the VHL products restricted with *BstUI*(+) or not restricted (-). Panel E shows the products of MSP for *E-cadherin*.

The cyclin-dependent kinase inhibitor *p15* is aberrantly methylated in many leukemic cell lines and 20 primary leukemias (Herman, et al., *supra*). For *p15*, MSP again verified the methylation status determined by Southern analysis. Thus, normal lymphocytes and cancer cell lines SW48 and U1752, all unmethylated by Southern analysis (Herman, et al., *supra*), only amplified with the 25 unmethylated set of primers, while the lung cancer cell line H1618 and leukemia cell line KG1A amplified only with the methylated set of primers (Fig. 3, panel A), consistent with previous Southern analysis results (Herman, et al., *supra*). The cell line Raji produced a 30 strong PCR product with methylated primers and a weaker band with unmethylated primers. This was the same result for methylation obtained previously by Southern analysis (Herman, et al., *supra*). Non-cultured leukemia samples,

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like the primary tumors studied for p16, had amplification with the methylated primer set as well as the unmethylated set. This heterogeneity also matched Southern analysis (Herman, et al., *supra*). Again, as for 5 p16, differential modification of *BstUI* restriction sites in the amplified product of p15 was used to verify the specific amplification by MSP (Fig. 3, panel B). Amplified products using methylated primer sets from cell lines H1618 and Raji or unmodified primer sets, were 10 completely cleaved by *BstUI*, while unmethylated amplified products did not cleave. Primary AML samples, which again only demonstrated cleavage in the methylated product, had less complete cleavage. This suggests a heterogeneity in methylation, arising because in some alleles, many CpG 15 sites within the primer sequences area are methylated enough to allow the methylation specific primers to amplify this region, while other CpG sites are not completely methylated.

Aberrant CpG island promoter region methylation is 20 associated with inactivation of the VHL tumor suppressor gene in approximately 20% of clear renal carcinomas (Herman, et al., *Proc. Natl. Acad. Sci. USA*, 91:9700, 1994). This event, like mutations for VHL (Gnarra, et al., *Nature Genetics*, 7:85, 1994), is restricted to clear 25 renal cancers (Herman, et al., *supra*). Primers designed for the VHL sequence were used to study DNA from the renal cell cancer line RFX393 which is methylated at VHL by Southern analysis, and the lung cancer cell line U1752 which is unmethylated at this locus (Herman, et al., 30 *supra*). In each case, the methylation status of VHL determined by MSP confirmed that found by Southern analysis (Fig. 3, panel C), and *BstUI* restriction site

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analysis validated the PCR product specificity (Fig. 3, panel D).

The expression of the invasion/metastasis suppressor gene, *E-cadherin*, is often silenced by 5 aberrant methylation of the 5' promoter in breast, prostate, and many other carcinomas (Graff, et al., *supra*; Yoshira, et al., *Proc. Natl. Acad. Sci. USA*, 92:7416, 1995). Primers were designed for the *E-cadherin* promoter region to test the use of MSP for this gene. In 10 each case, MSP analysis paralleled Southern blot analysis for the methylation status of the gene (Graff, et al., *supra*). The breast cancer cell lines MDA-MB-231, HS578t, and the prostate cancer cell lines DuPro and TSUPrI, all heavily methylated by Southern, displayed prominent 15 methylation. MCF7, T47D, PC-3, and LNCaP, all unmethylated by Southern, showed no evidence for methylation in the sensitive MSP assay (Fig. 3, panel E). MSP analysis revealed the presence of unmethylated alleles in Hs578t, TSUPrI and DuPro consistent with a low 20 percentage of unmethylated alleles in these cell lines previously detected by Southern analysis (Graff, et al., *supra*). *Bst*UI restriction analysis again confirmed the specificity of the PCR amplification.

Table 1 PCR primers used for Methylation Specific PCR

Primer	Sense primer*	Antisense primer*	Size (bp)	Anneal temp.	Genomic Position†
Set	(5'-3')	(5'-3')			
p16-W	CAGAGGGTGGGGGACCGG	CGGGCGGGGGTGG	140	65°C	+171
p16-M	TTATTAGGGTGGGGGAT <u>GC</u>	<u>GACCCG</u> A <u>CCG</u> GACCG <u>TAA</u>	150	65°C	+167
p16-U	TTATTAGGGTGGGG <u>TGT</u>	C <u>A</u> CC <u>CC</u> <u>AA</u> AC <u>CC</u> <u>AC</u> <u>CC</u> <u>AT</u> <u>AA</u>	151	65°C	+167
p16-M2	TTATTAGGGTGGGG <u>GG</u> <u>AT</u> <u>GC</u>	CCACCTAAAT <u>CG</u> <u>AC</u> <u>CT</u> <u>CC</u> <u>GC</u> <u>CG</u>	234	65°C	+167
p16-U2	TTATTAGGGTGGGG <u>TG</u> <u>GT</u> <u>T</u>	CCACCTAAAT <u>CA</u> <u>CC</u> <u>TC</u> <u>CC</u> <u>AA</u> <u>CC</u> <u>AA</u>	234	60°C	+167
p15-W	CGCACCTGGGGCAGA	AGTGGCGAGGGGGGG	137	65°C	+46
p15-M	<u>GG</u> <u>TT</u> <u>GT</u> <u>AT</u> <u>TT</u> <u>TC</u> <u>GG</u> <u>TT</u>	<u>CG</u> <u>T</u> <u>AC</u> <u>AA</u> <u>TA</u> <u>AC</u> <u>CG</u> <u>AC</u> <u>CC</u> <u>GA</u>	148	60°C	+40
p15-U	<u>T</u> <u>G</u> <u>T</u> <u>A</u> <u>G</u> <u>T</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>GG</u> <u>TT</u>	<u>CC</u> <u>A</u> <u>TA</u> <u>CA</u> <u>AA</u> <u>TA</u> <u>AC</u> <u>CC</u> <u>AA</u> <u>AC</u> <u>CC</u> <u>AA</u>	154	60°C	+34
VHL-M	TGGAGGATTTTTGGT <u>AC</u> <u>CC</u>	<u>GA</u> <u>CC</u> <u>GA</u> <u>AC</u> <u>CC</u> <u>GG</u> <u>GA</u>	158	60°C	-116
VHL-U	GTGGAGGATTTTT <u>TG</u> <u>GT</u> <u>AT</u> <u>GT</u> <u>T</u>	<u>CC</u> <u>CA</u> <u>AA</u> <u>CC</u> <u>AA</u> <u>AC</u> <u>CC</u> <u>AA</u> <u>AA</u>	165	60°C	-118
Ecad-M	TTAGGTAGGGTTATCGGT	TA <u>CT</u> <u>AA</u> <u>AA</u> <u>TT</u> <u>CA</u> <u>CT</u> <u>AC</u> <u>CC</u> <u>GA</u>	116	57°C	+205
Ecad-U	TA <u>AT</u> <u>TT</u> <u>TA</u> <u>GG</u> <u>TT</u> <u>AG</u> <u>GG</u> <u>TT</u> <u>AT</u> <u>TC</u> <u>T</u>	CACAA <u>CC</u> <u>AA</u> <u>AT</u> <u>CA</u> <u>CA</u> <u>AC</u> <u>AC</u> <u>CA</u> <u>CA</u>	97	53°C	-210

*Sequence differences between modified primers and unmodified DNA are boldface, and differences between methylated/modifited and unmethylated/modifited are underlined.
†Primers were placed near the transcriptional start site. Genomic position is the location of the 5' nucleotide of the sense primer in relation to the major transcriptional start site defined in the following references and Genbank accession numbers: p16(most 3' site) X9154 (E. Hara, et al., Mol. Cell Biol., 16:459, 1996), p15 S7576 (J. Jen, et al., Cancer Res., 54: 6153 1994), VHL U19763 (I. Kuzmin, et al., Oncogene, 10:2185 1995), and E-cadherin L34545 (M. J. Busschakers, et al., Biochem. Biophys. Res. Commun. 203: 1284 1994).

†W represents unmodified, or wild-type primers, M represents methylated-specific primers, and U represents unmethylated-specific primers.
(SEQ ID NO:105-128)

Table 2

Gene	Sense Primer	Antisense Primer	PCR Product	Temp	Genbank#	Position	
Androgen	Me	5'gggttttaggttatttagagggtgc	5'accaaataaccataaaaccccttacg	~210bp	55°C	M27423 189	
Receptor	Un	5'gggttttaggttatttagagggtt	5'accaaataaccataaaaccccttaca	~210bp	55°C	M27423 189	
5	Estrogen	Me	5'gtgttattggatagttagtaagtgtc	5'cgtaaaaaaaaccgtttaacgtaaa	118bp	57°C	X62462 3127
	Receptor	Un	5'gggttattggatagttagtaagtgtt	5'ccataaaaaaaaccaatctaacc	120bp	57°C	X62462 3126
MDG1	Me	5'ttttttcgggaggcgcccc	5'aacgtacaaaaactccggacgacg	105bp	60°C	U57623 1134	
	Un	5'ttttttattttggatgtgggggt	5'aaacatacaaaaactccaaacaaca	113bp	60°C	U57623 1131	
GS	Me	5'ttccccgggttagcggtcgtc	5'gcccaatactaaatcacgcacg	91bp	60°C	X08058 1082	
	Un	5'gatgttgggggtatgtgggttt	5'ccacccaaatactaaaatcacaca	97bp	60°C	X08058 1078	
10	Calci-tonin	Me	5'cgggaaataagagtagtgcgtggc	5'caaaaccctataaaaaacgacgacg	84bp	60°C	X15943 1801
	Un	5'ggggaaataagagtagtgcgttgt	5'ccaaaaccctataaaaaacaaacaaca	87bp	60°C	X15943 1799	
HIC-1	Me	5'gtcgtacgtcggttgcgtc	5'gatacccgccctaacgcgg	259bp	60°C		
	Un	5'gggttgtatgtgggttgttt	5'cacaatacccccctaacacca	264bp	60°C		
15							

Example 4

Indirect *in situ* p16 Methylation. Indirect *in situ* PCR (indirect IS-PCR) describes a technique whereby the 5 amplicon is produced by thermal cycling without label incorporation. At the end of the cycling reaction, the amplified product is detected by standard *in situ* hybridization using a labeled probe.

Sample Preparation and Pretreatment. Paraffin-10 embedded formalin fixed HN12 cells were deparaffinized in xylene for 5 minutes, followed by two 5 minute rinses in 100% ethanol. Slides were air dried, and then treated with Proteinase K(10 μ g/ml in 50mM Tris) for 30 minutes at 37°C under a coverslip. Slides were rinsed in distilled 15 water and placed in 0.2N NaOH for 10 minutes at 37°C, then placed in 3M Sodium bisulfite containing hydroxyquinone. The solution was layered with mineral oil and left at 50°C for 16 hours. The oil was removed, and the slides were rinsed in water and placed in 0.3M NaOH for 5 minutes at 20 room temperature. Slides were then dehydrated in 70%, 80%, and 100% ethanol and air dried.

Indirect IS-PCR Protocol. The PCR mixture contained 1xPCR buffer (16.6mM ammonium sulfate/67mM Tris, pH 8.8/6.7mM MgCl₂/10mM 2-mercaptoethanol), dNTPs 25 (2.5 lambda of 25mM mix), 300ng of each primer, and 2.5 Units of Taq Polymerase per 50 μ l reaction. This mixture was "hot started" at 95°C for 5 minutes, then added directly to the slides which were prewarmed to 80°C with a GeneComb in place. PCR was performed on a Hybaid 30 Omniprep Cycler with an *in situ* module. Thirty cycles of 90°C for 30 seconds, 58°C for 45 seconds, and 70°C for 45 seconds were performed with a calibration number of 50. After the PCR, slides were immediately rinsed once in

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distilled water, followed by dehydration in 70%, 80%, and 100% ethanol and air dried.

Indirect IS-PCR In Situ Hybridization and Fluorescence Detection. Following the PCR, the specimen 5 was fixed briefly to maintain the localization of the PCR product. This was accomplished by fixing the sections in 100% ethanol for 15 minutes and then allowing them to air dry before application of the probe. A methylated p16 PCR product was generated and labeled with digoxigenin, 10 and used as a labeled probe for *in situ* hybridization. The digoxigenin labeled probe was suspended in a hybridization buffer containing 2xSSC/10% Dextran Sulfate/50% Formamide for a final concentration of 10ng/ul. 10.0 μ l(100.0ng of probe) of the hybridization 15 mixture was placed on each sample, covered with a glass coverslip and sealed with rubber cement. The cell preparations were then incubated at 95°C for 5 minutes and hybridized at 37°C overnight in a humidified chamber.

Following hybridization, the HN12 samples were 20 post-washed in 2xSSC (salt sodium citrate) at room temperature for five minutes and placed in 1xPBD (phosphate buffered detergent) at room temperature prior to immunological detections.

All hybridized HN12 cell samples were 25 immunocytochemically stained with fluorescein labeled avidin (Vector Laboratories, CA). Sixty microliters of detection reagent, consisting of fluorescein labeled avidin (10ug/ml), 1XPBS, 5% powdered dry milk, and 0.02% sodium azide was applied to the slide under a plastic 30 coverslip and incubated at 37°C for five minutes in a humidified chamber. The slides were washed in 1XPBD several times and counterstained with propidium iodide (0.3ug/ml) in antifade solution.

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For fluorescence microscopy, a Zeiss Axiophot 20 epi-fluorescence microscope (Zeiss, West Germany) equipped with a 100-Watt mercury-arc lamp, a 100X Plan-Neofluar oil immersion objective (Zeiss, West 5 Germany), a Zeiss MC-100 camera (Zeiss, West Germany), and the appropriate filter sets for fluorescein (FITC)/PI fluorescence (Chroma, VT) were utilized. Image analysis and record keeping was performed using an OIS (Oncor Instrument Systems) 3CCD Cooled Camera System and OIS 10 2.01 Image Analysis Software.

The negative PCR control HN12 cell sample was processed using an initial bisulfite reaction to modify the DNA, followed by PCR amplification using primers specific for the methylated DNA of the promoter regions 15 of the p16 gene, and *in situ* hybridization. The negative control displayed no visible signal as expected, since the PCR reaction was incomplete.

P16 methylated HN12 cells were subjected to the bisulfite modification, PCR amplification using primers 20 specific for the methylated DNA of the promoter regions of the p16 gene, and *in situ* hybridization using the labeled probe. Fluorescent signals specific for the amplified, localized product of the methylated p16 promoter regions were visible in the cells.

25 P16 methylated HN12 cells were subjected to the bisulfite modification, PCR amplification using primers specific for the unmethylated DNA of the promoter regions of the p16 gene, and *in situ* hybridization using the labeled probe. No fluorescent signals were visible in 30 the cells.

Example 5

Testing the Use of MSP for Methylation

Changes in Sputum Samples. MSP has been tested to detect

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methylation changes in DNA from sputum samples. The samples tested included the primers for p16 (Table 1; SEQ ID NOS:105-110) and DNA extracted from sputum samples from two patients known to have lung cancer. The 5 unmethylated alleles for p16 were detected, however, methylated alleles were not detected. It appears that since the MSP worked with the non-methylated alleles, that the patient's tumors did not have methylated p16. To test this hypothesis, one sputum sample was spiked 10 with cells from the established lung cancer cell line Calu 3, which has a hypermethylated p16 gene. These p16 methylated alleles were easily detected in this mixed DNA.

Example 6**MSP for detection of hMLH1 and hMSH2**

hypermethylation. The hMLH1 and hMSH2 genes encode for mismatch repair proteins, and mutations in each can cause 5 inherited forms of colon cancer marked by microsatellite instability. Some 20% of patients with non-inherited colon cancer also have tumors with the microsatellite instability phenotype. The precise mechanism causing these latter tumors is not clear. Kane et al. (Cancer 10 Research, 57:808, 1997) have reported in small series, that tumors from patients with the sporadic form of colon cancers with microsatellite instability have hypermethylation of the promoter of the hMLH1 gene. MSP, employing the primer set and conditions for hMSH2 and 15 hMLH1 (Table 2; SEQ ID NO:161-172), was used to follow up on this observation in a larger series of tumors. In some 14 patients with such tumors, 85% have hypermethylation of hMLH1 while only 5% of 21 colon tumors without microsatellite instability show this 20 change. No hypermethylation of hMSH2 was seen in either group. The data indicate that 15% or so of all patients with colon cancer, who are known to have tumors with microsatellite instability, have hypermethylation of the hMLH1 gene in tumor DNA, and that transcriptional 25 silencing associated with this change is the cause of the genetic instability in this setting.

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Example 7

MSP studies of the Tissue Inhibitor of Metalloproteinase II (TIMP 2) gene. TIMP2 is a member of a family of metalloproteinase inhibitors which are 5 necessary to limit the invasive potential of multiple cell types. Using the MSP primers and conditions for this gene (Table 2; SEQ ID NOS:157-160), hypermethylation of the TIMP2 promoter was found in approximately 50% of primary colon cancer. This change, and the associated 10 loss of expression for this gene, could be a key factor in increasing the invasive potential for the colon cancers involved.

Example 8

MSP Studies of the TGF-beta Receptor I and II genes. MSP primers and conditions previously provided (Table 2; SEQ ID NOS:177-184) have been successfully employed for TGF-beta receptor I and II genes, and no evidence for promoter hypermethylation in tumors has been found to date.

20

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A method for detecting a methylated CpG-containing nucleic acid comprising:
5 contacting a nucleic acid-containing specimen with an agent that modifies unmethylated cytosine, amplifying the CpG-containing nucleic acid in the specimen by means of CpG-specific oligonucleotide primers, wherein the oligonucleotide primers distinguish between modified methylated and non-methylated nucleic acid and detecting the methylated nucleic acid.
10
2. The method of claim 1, wherein the amplifying step is the polymerase chain reaction (PCR).
15
3. The method of claim 1, wherein the modifying agent is bisulfite.
20
4. The method of claim 1, wherein cytosine is modified to uracil.
25
5. The method of claim 1, wherein the CpG-containing nucleic acid is in a promoter region.
6. The method of claim 5, wherein the promoter is a tumor suppressor gene promoter.
7. The method of claim 6, wherein the tumor suppressor gene is selected from the group consisting of p16, p15, E-cadherin, and VHL.
25

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8. The method of claim 1, wherein the CpG-containing nucleic acid encodes a protein selected from the group consisting of androgen receptor, estrogen receptor, TGF-beta1, TGF-beta2, p130, BRCA2, NF1, NF2, TSG101, MDGI, GST-pi, calcitonin, HIC-1, endothelin B receptor, TIMP-2, 06-MGMT, MLH1, MSH2, and GFAP.
5
9. The method of claim 1, wherein the specimen is selected from the group consisting of brain, colon, urogenital, lung, renal, hematopoietic, breast, thymus, testis, ovarian, and uterine tissue or serum, urine, saliva, cerebrospinal fluid, sputum, ejaculate, blood, and stool.
10
15
10. The method of claim 1, further comprising contacting the nucleic acid with a methylation sensitive restriction endonuclease.
20
11. The method of claim 10, wherein the restriction endonuclease is selected from the group consisting of *Msp*I, *Hpa*II, *Bss*HII, *Bst*UI and *Not*I.
25
12. The method of claim 1, wherein the presence of methylated CpG-containing nucleic acid in the specimen is indicative of a cell proliferative disorder.
13

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13. The method of claim 12, wherein the disorder is selected from the group consisting of low grade astrocytoma, anaplastic astrocytoma, glioblastoma, medulloblastoma, colon cancer, 5 lung cancer, renal cancer, leukemia, breast cancer, prostate cancer, endometrial cancer and neuroblastoma.
14. The method of claim 1, wherein the primer hybridizes with a target polynucleotide sequence having the sequence selected from the group consisting of SEQ ID NO:1-103 and 10 SEQ ID NO:104.
15. The method of claim 1, wherein the primers are selected from the group consisting of SEQ 15 ID NO:105-207 and SEQ ID NO:208.
16. A kit useful for the detection of a methylated CpG-containing nucleic acid comprising carrier means being compartmentalized to receive in close 20 confinement therein one or more containers comprising a first container containing a reagent which modifies unmethylated cytosine.
17. The kit of claim 16, wherein the kit further comprises a second container containing 25 primers for amplification of the CpG-containing nucleic acid.
18. The kit of claim 16, wherein the reagent is bisulfite.

19. The kit of claim 16, wherein cytosine is modified to uracil.
20. The kit of claim 17, wherein the primer hybridizes with a target polynucleotide sequence having the sequence selected from the group consisting of SEQ ID NO:1-103 and SEQ ID NO:104.
5
21. The kit of claim 17, wherein the primers are selected from the group consisting of SEQ ID NO:105-207 and SEQ ID NO:208.
10
22. A kit for the detection of methylated CpG-containing nucleic acid from a sample comprising:
 - 15 a) a reagent that modifies unmethylated cytosine nucleotides;
 - b) control nucleic acid;
 - c) primers for the amplification of unmethylated CpG-containing nucleic acid;
 - 20 d) primers for the amplification of methylated CpG-containing nucleic acid; and
 - e) primers for the amplification of control nucleic acid.
23. The kit of claim 22, further comprising
25 nucleic acid amplification buffer.
24. The kit of claim 22, wherein the reagent that modifies unmethylated cytosine is bisulfite.

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25. The kit of claim 22, wherein primers hybridize with a target polynucleotide sequence having the sequence selected from the group consisting of SEQ ID NO:1-103 and SEQ ID NO:104.
5
26. The kit of claim 22, wherein the primers are selected from the group consisting of SEQ ID NO:105-207 and SEQ ID NO:208.
10
27. Isolated oligonucleotide primer(s) for detection of a methylated CpG-containing nucleic acid wherein the primer hybridizes with a target polynucleotide sequence having the sequence selected from the group consisting of SEQ ID NO:1-103 and SEQ ID NO:104.
15
28. The primers of claim 27, wherein the primer pairs are SEQ ID NO:105-207 and SEQ ID NO:208, in sequential pairs.
20
29. A method for detecting a cell proliferative disorder associated with methylation of CpG islands in TIMP-2 nucleic acid a subject comprising:
25 contacting a target nucleic acid in a sample of tissue or biological fluid from the subject with a reagent which detects TIMP-2 wherein the reagent detects methylation of TIMP-2 when the target nucleic acid is DNA, and wherein the reagent detects the level of TIMP-2 RNA
30 when the target nucleic acid is RNA; and

detecting TIMP-2 target nucleic acid, respectively, wherein hypermethylation of the promoter of DNA, respectively, or decreased levels of TIMP-2 RNA, respectively, as compared with the level of TIMP-2 RNA 5 respectively, in a normal cell, is indicative of a TIMP-2-associated cell proliferative disorder.

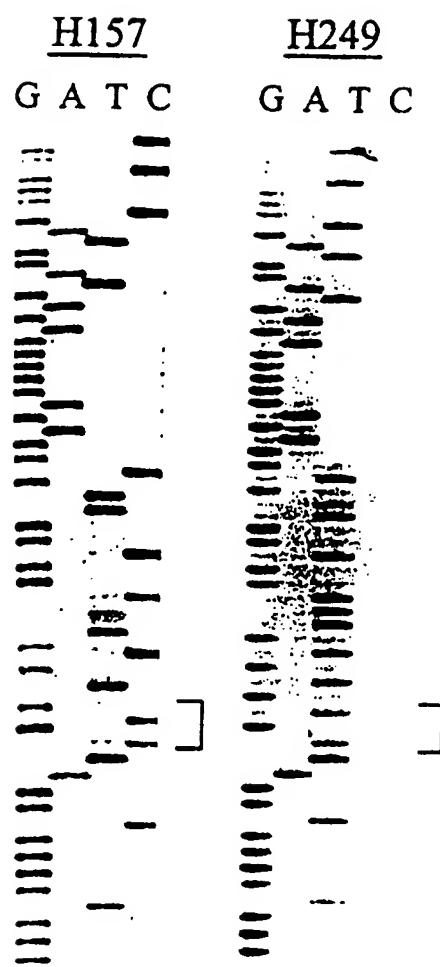
30. The method of claim 29, wherein the reagent is a nucleic acid probe.
31. The method of claim 30, wherein the probe is detectably labeled.
10
32. The method of claim 31, wherein the label is selected from the group consisting of radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.
15
33. The method of claim 29, wherein the reagent which detects methylation is a restriction endonuclease.
34. The method of claim 33, wherein the restriction endonuclease is methylation sensitive.
20
35. The method of claim 34, wherein the restriction endonuclease is selected from the group consisting of *Msp*I, *Hpa*II and *Bss*HII.
36. The method of claim 29, wherein the biological fluid is selected from the group consisting of serum, urine, saliva,
25

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cerebrospinal fluid, sputum, ejaculate,
blood, and stool.

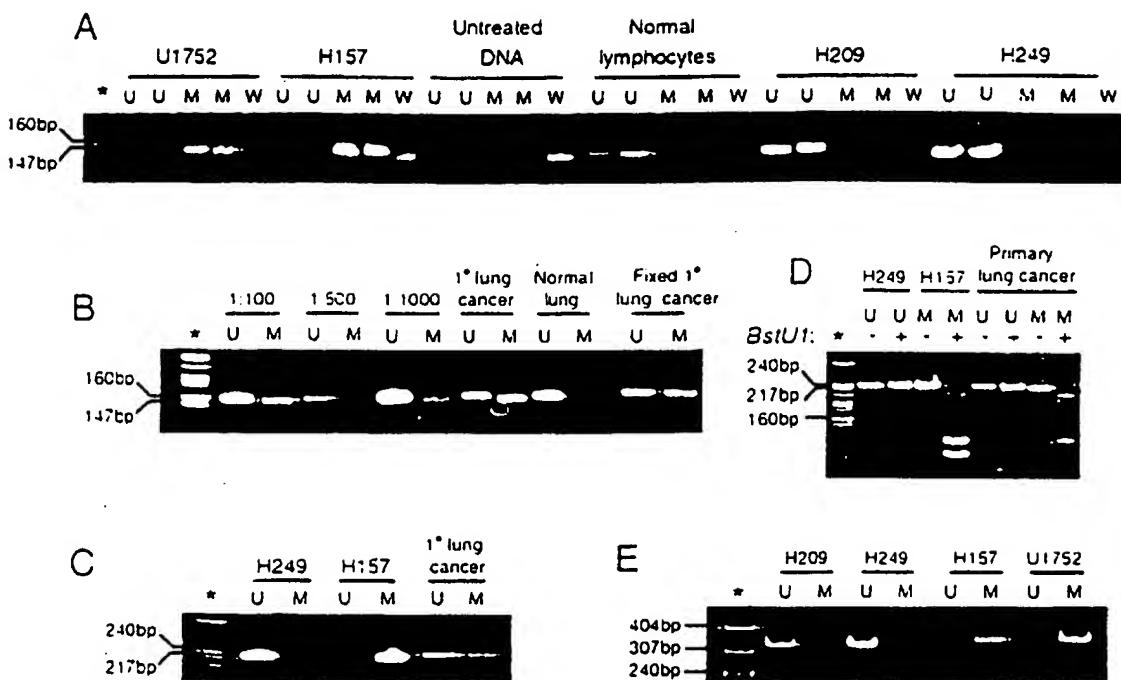
1/3

Figure 1



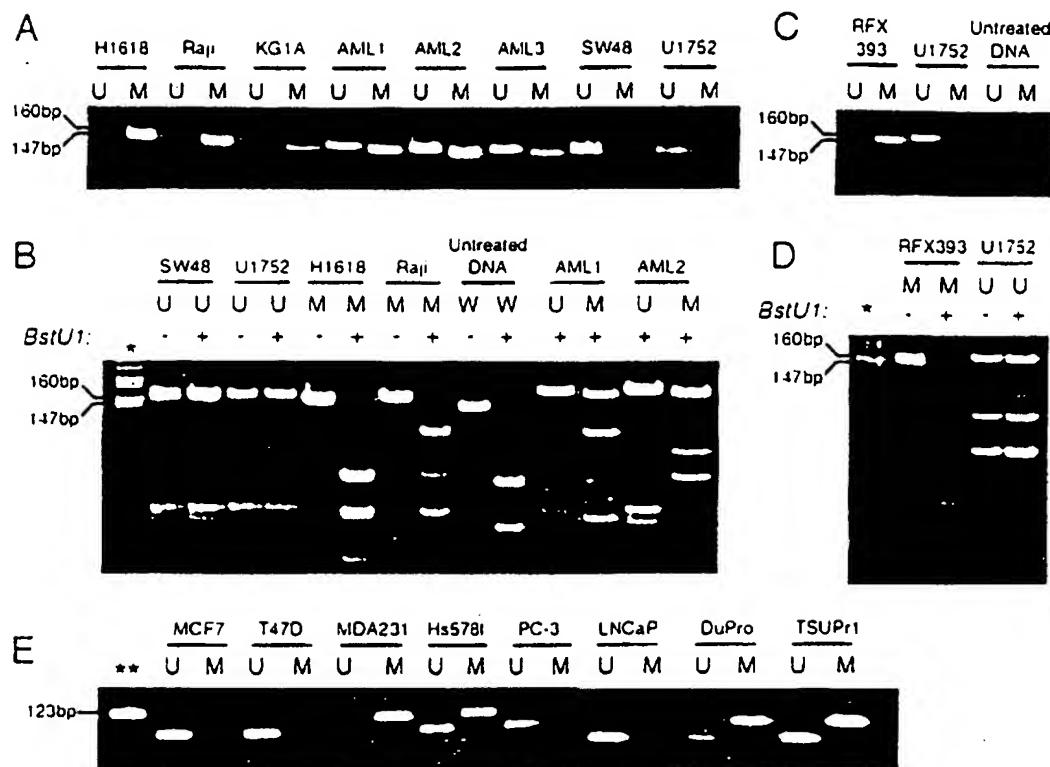
2/3

Figure 2



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Figure 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09533

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/04; C12P 19/34
US CL :435/6, 91.2; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA, DERWENT
search terms: methylation, PCR, primers, bisulfite, TIMP-2, RNA expression

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y ----	FROMMER et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc. Natl. Acad. Sci. USA. March 1992, Vol. 89, pages 1827-1831, especially pages 1828-1829.	16-19 and 22-24
A	STETLER-STEVENSON et al. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J. Biol. Chem. 15 August 1990, Vol. 265, No. 23, pages 13933-13938.	1-15, 20, 21, and 25- 28
A	US 5,324,634 A (ZUCKER) 28 June 1994	29-36
A,P	US 5,595,885 A (STETLER-STEVENSON et al.) 21 January 1997.	29-36

Further documents are listed in the continuation of Box C.

See patent family annex.

•	Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
•A*	document defining the general state of the art which is not considered to be of particular relevance		
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•L*	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
•O*	document referring to an oral disclosure, use, exhibition or other means	*&*	document member of the same patent family
•P*	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

04 AUGUST 1997

Date of mailing of the international search report

29 AUG 1997

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